G-PROTEIN-COUPLED RECEPTORS

GPCR STRUCTURE AND FUNCTION

G-protein-coupled receptors (GPCRs) or seven transmembrane domain receptors (7TM) comprise the largest superfamily of proteins in the body. In vertebrates, this family contains 1000–2000 members (more than the 1% of the genome) including more than 1000 coding for odorant and pheromone receptors. The chemical diversity among the endogenous ligands is exceptional; they include biogenic amines, peptides, glycoproteins, lipids, nucleotides and ions (Kolakowski, 1994). Moreover, the sensation of exogenous stimuli, such as light, odors, and taste, is mediated via this class of receptors (Hoon et al., 1999). It has been estimated that more than half of all modern drugs are targeted at these receptors (Flower, 1999), and several ligands for GPCRs are found among the worldwide top-100-selling pharmaceutical products. It is also evident that drugs have still only been developed to affect a very small number of the GPCRs, and the potential for drug discovery within this field is enormous.

There are two main requirements for a protein to be classified as a GPCR. The first requirement relates to seven sequence stretches of about 25 to 35 consecutive residues that show a relatively high degree of calculated hydrophobicity. These sequences are believed to represent seven α-helices that span the plasma membrane in a counter-clockwise manner, forming a recognition and connection unit, enabling an extracellular ligand to exert a specific effect into the cell. The second principal requirement is the ability of the receptor to interact with a G-protein. As shown in Figure 1, GPCRs act at the heterotrimeric G proteins as guanine-nucleotide exchange factors; thus, the activated receptor induces a conformational change in the associated G protein α-subunit leading to release of GDP followed by binding of GTP. Subsequently, the GTP-bound form of the α-subunit dissociates from the receptor as well as from the stable βγ-dimer (Marinissen, 2001). Both the GTPbound α-subunit and the released βγ-dimer can modulate several cellular signaling pathways. These include, among others, stimulation or inhibition of adenylyl cyclases and activation of phospholipases, as well as regulation of potassium and calcium channel activity (Hamm, 1998). The complexity of GPCR signaling has recently been further underlined by data indicating that GPCRs may not solely act via heterotrimeric G proteins (Daaka et al., 1998; Lefkowitz, 1998). Most intriguingly, it has been suggested that agonist-promoted phosphorylation of the receptors by GRKs (G protein-coupled receptor kinases) and subsequent sequestration of the receptors from the cell surface (Krupnick and Benovic, 1998) are not only important for turning off signaling, but also play a key role in switching the receptor from G protein-dependent pathways to G-protein independent signaling cascades normally used by growth factor receptors (Luttrell et al., 1999).

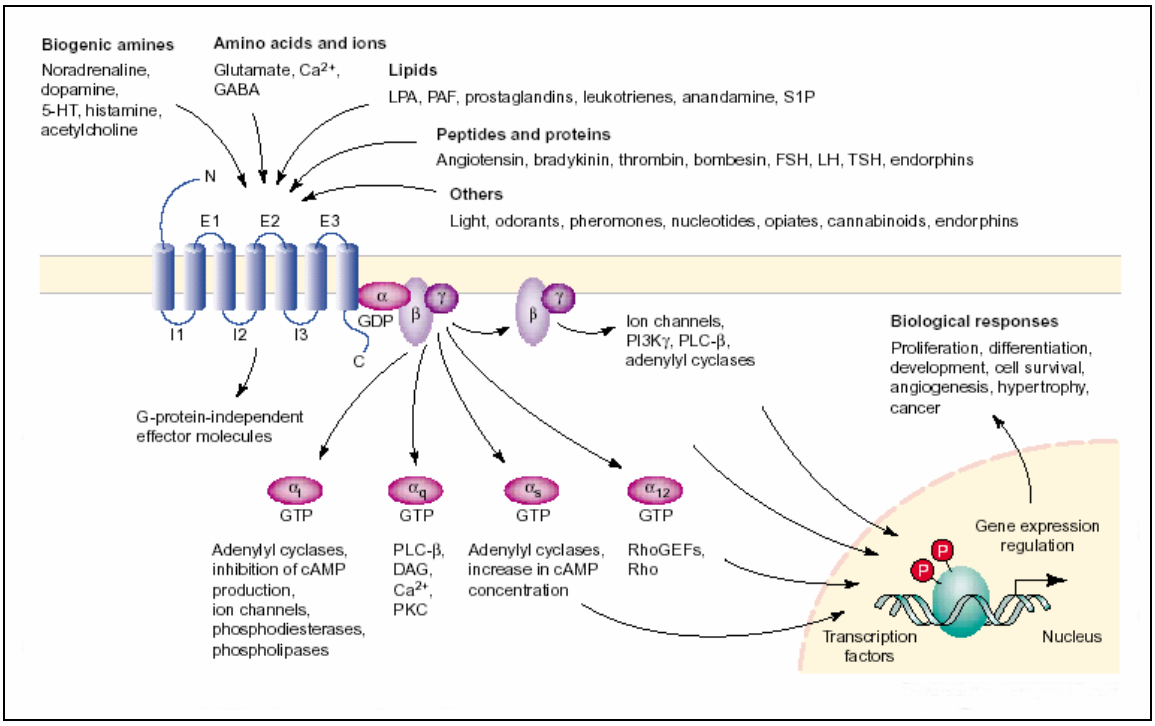


Figure 1: Diversity of G-protein-coupled receptors (GPCRs). A wide variety of ligands use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G-protein-dependent and -independent pathways. Such signaling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. Abbreviations: DAG, diacylglycerol; FSH, follicle-stimulating hormone; GEF, guanine nucleotide exchange factor; LH, leuteinizing hormone; LPA, lysophosphatidic acid; PAF, plateletactivating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine-1-phosphate; TSH, thyroid-stimulating hormone.

The exposure of GPCRs to agonists often results in a rapid attenuation of receptor responsiveness. This process, termed desensitization, is the consequence of a combination of different mechanisms. These mechanisms include the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation (Hausdorff et al., 1989; Lohse et al., 1990), the internalization of cell surface receptors to intracellular membranous compartments (Hermans et al., 1997; Trejo et al., 1998) and the downregulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis, as well as both the lysosomal and plasma membrane degradation of pre-existing receptors (Jockers et al., 1999; Pak et al., 1999). The time frames over which these processes occur range from seconds (phosphorylation) to minutes (endocytosis) and hours (down-regulation) and the extent of receptor desensitization varies from complete termination of signaling, as observed in the visual and olfactory systems, to the attenuation of agonist potency and maximal

responsiveness, such as observed for the β_2-adrenergic receptor (Sakmar, 1998). However, the extent of receptor desensitization is regulated by a number of factors that include receptor structure and cellular environment.

The most rapid means by which GPCRs are uncoupled from heterotrimeric G proteins is through the covalent modification of the receptor as a consequence of phosphorylation by intracellular kinases. It is generally accepted that both second messenger-dependent protein kinases [cAMP-dependent protein kinase (PKA) and protein kinase C (PKC)] and GRKs (*G-protein coupled receptor kinases*) phosphorylate serine and threonine residues within the intracellular loop and carboxyl-terminal tail domains of GPCRs (Lefkowitz, 1993; Krupnick and Benovic, 1998). Second messenger-dependent protein kinases not only phosphorylate agonist-activated GPCRs, but also indiscriminately phosphorylate receptors that have not been exposed to agonist (Hausdorff et al., 1989). In contrast, GRK family members selectively phosphorylate agonist activated receptors, thereby promoting the binding of cytosolic cofactor proteins called arrestins, which sterically uncouple the receptor from heterotrimeric G proteins (Lohse et al., 1990). The GRK family of kinases is comprised of seven family members (GRK1-7). GRK1 (or rhodopsin kinase), GRK2 (or βark1, *β-adrenergic receptor kinase*), GRK3 (or βark2) and GRK7 are cytosolic, whereas GRK4, GRK5 and GRK6 are associated to the plasma membrane by some lipidic modification of their amino acids.

Internalization of G protein-coupled receptors is a commonly observed response following agonist-stimulation. Over the years, numerous reports have addressed the role of receptor sequestration in desensitization and resensitization. While some evidence suggests that this phenomenon is part of the desensitization process, other evidence suggests that internalization is instead one of the means by which receptors are resensitized. Indeed, trafficking of an uncoupled receptor to endosomal compartments allows dephosphorylation and recycling of the receptor to the cell surface (Krueger et al., 1997). This is in contrast to receptor “down-regulation” observed after prolonged agonist exposure, which leads to targeting of the receptors to the degradation pathway (Bohm et al., 1997). GRKs and ß-arrestins appear to be key regulatory molecules for receptor internalization since these proteins have been shown to interact with components of the clathrin-coated vesicle pathway. In response to GPCR activation, cytosolic ß-arrestin proteins translocate to the plasma membrane and then subsequently redistribute to clathrin/coated pits bound to receptors. The clathrin-coated vesicle pathway is the best characterized endocytic route and is utilized by constitutively recycling receptors, by tyrosine kinase receptors, and by numerous heptahelical or G protein-coupled receptors. Clathrin is a trimeric protein arranged as a triskelion when assembled and is the major structural protein of the characteristic polygonal lattice of the coated pit. Plasma membrane coated pits also contain the clathrin adaptor protein AP-2, which binds to ß-arrestins and to clathrin (Figura 2).

Nevertheless, not all GPCRs necessarily internalize in a ß-arrestin- and clathrin-dependent manner. Experimental evidence suggests that G protein-coupled receptors can be internalized via alternative endocytic pathways. Some G protein-coupled receptors have been found in cholesterol rich plasma membrane structures termed caveolae (Chun et al., 1994; Huang et al., 1997; Burgueno et al., 2003a)a). These domains are also known as signaling domains, but appear to

contain proteins involved in the formation and budding of vesicles such as the dynamin molecule. The use of biochemical agents known to disrupt these structures has been effective in modifying the endocytosis of certain G protein-coupled receptors (Gines et al., 2001; Escriche et al., 2003). Finally, some receptors are suspected to use a third alternative endocytic pathway. No coat or adaptor proteins have been identified for the generation of these vesicles (Claing et al., 2000).

Once internalized, receptors are targeted to recycling or degradative pathways. Some G protein-coupled receptors, including the ß2-adrenergic receptor, can be recycled back to the plasma membrane, as a fully competent receptor within minutes of being internalized (Pippig et al., 1995). Other receptors such as the vasopressin type 2 receptor are detained within the cell for longer time period before being recycled back to the cell surface (Innamorati et al., 2001), while others like the δ-opioid or thrombin receptors are degraded (Tsao and von Zastrow, 2000).

However, GPCR desensitization and endocytosis can act as molecular switches coupling GPCRs to alternative signal transduction pathways. ß-Arrestins not only function in the molecular switch required for GPCR desensitization and internalization, but also act as scaffolds to transduce and compartmentalize the alternative signals. In fact, ß-arrestins have the ability to interact with a variety of endocytic as well as signaling proteins such as c-Src (Luttrell et al., 1999), MAPKs and Raf (DeFea et al., 2000).

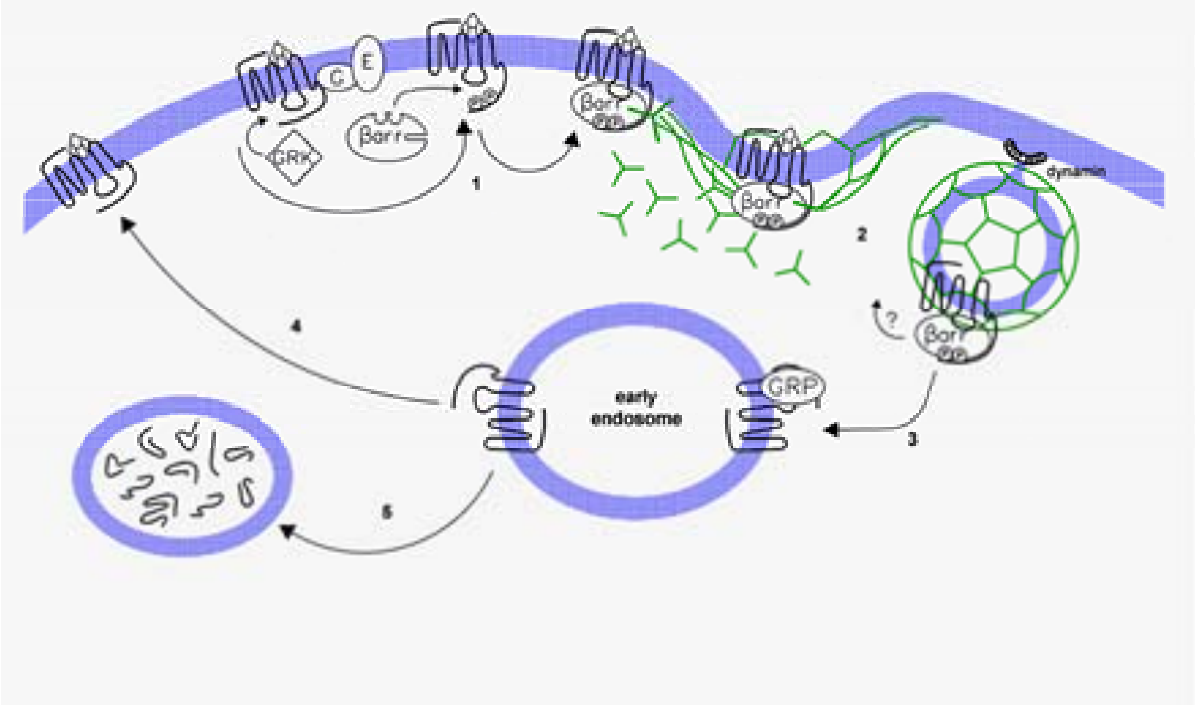


Figure 2: Desensitization, internalization, down-regulation and resensitization model proposed for the GPCRs. 1-. G-protein uncoupling and clathrin binding. 2-. Coated reversion. 3-. Endocytosis. 4-. Recycling. 5-.. Degradation in the lysosome Abbreviations: GRK, GPCR kinases, βarr, β-arrestin, G, G-protein, E, effector system, GRP, GPCRs phosphatase.

The first crystal structure of a GPCR appeared in the year 2000, when Palczewski et al.(Palczewski et al., 2000), reported the high-resolution structure for the bovine rhodopsin receptor. With 2.3 Á resolution, it was confirmed that the α-helical transmembrane domains (TMD) are arranged in a closely packed bundle forming the transmembrane receptor core. The N-terminus of the polypeptide is located in the extracellular space whereas the C terminus shows an intracellular localization. The seven transmembrane helices are connected by six alternating intracellular (ICL) and extracellular (ECL) loops. Nevertheless, the current rhodopsin model still lacks detailed structural information of some parts of the connecting loops and the C-terminus.

Like other membrane proteins, GPCRs are partially buried in the non-polar environment of the lipid bilayer by forming a compact bundle of transmembrane helices. The correct orientation and integration of the polypeptide chain is guided by a complex translocation apparatus residing in the ER. Two different folding stages can be distinguished following an initial translocation of the receptor N terminus into the ER lumen. In the first stage, hydrophobic α-helices are established across the lipid bilayer, and protein folding is predominantly driven by the hydrophobic effect. The TMDs adopt a secondary structure in order to minimize the polar surface area exposed to the lipid environment with the result that hydrophobic amino acids face the lipid bilayer and that more hydrophilic amino acid residues are orientated towards the core crevice of the TMD bundle. Finally, in the second stage, a functional tertiary structure is formed by establishing specific helix-helix interactions, leading to the tightly packed, ring-like structure of the TMD bundle.

GPCR CLASSIFICATION

GPCRs do not share any overall sequence homology, the only structural feature common to all GPCRs is the presence of seven transmembrane-spanning α-helical segments connected by alternating intracellular and extracellular loops. Two cysteine residues (one in ECL 1 and one in ECL 2) which are conserved in most GPCRs, form a disulfide link which is probably important for the packing and for the stabilization of a restricted number of conformations of these seven transmembrane domains. Aside from sequence variations, GPCRs differ in the length and function of their N-terminal extracellular domain, their C-terminal intracellular domain and their intracellular loops. Each of these domains provides specific properties to these various receptor proteins. However, significant sequence homology is found, within several subfamilies (Attwood and Findlay, 1994). The three major subfamilies include the receptors related to the “light receptor” rhodopsin and the β2-adrenergic receptor (family A), the receptors related to the glucagon receptor (family B), and the receptors related to the metabotropic neurotransmitter receptors (family C). Yeast pheromone receptors make up two minor unrelated subfamilies, family D (STE2 receptors) and family E

(STE3 receptors). Finally, in *Dictyostelium Discoideum* four different cAMP receptors constitute yet another minor, but unique, subfamily of GPCRs (family F) (Kolakowski, 1994). A schematic representation is shown in Figure 3.

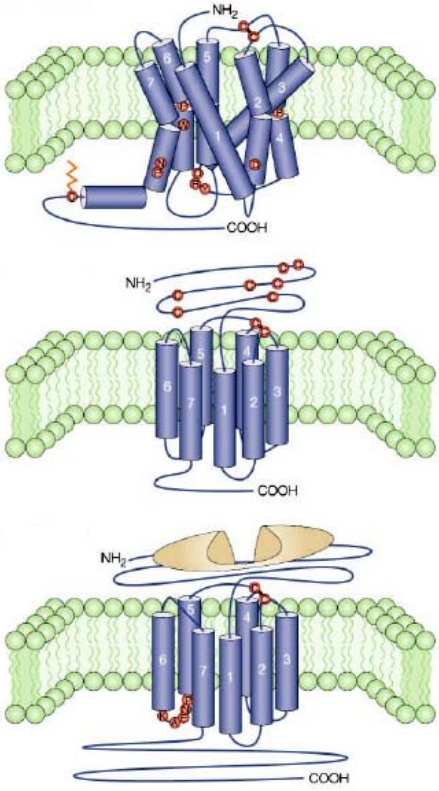
The family A receptors, that comprises the rhodopsin/β2 adrenergic receptor-like receptors, contains the 90% of all GPCRs and is by far the largest and the most studied. The overall homology among all type A receptors is low and restricted to a number of highly conserved key residues. The high degree of conservation among these key residues suggests that they have an essential role for either the structural or functional integrity of the receptors. The only residue that is conserved among all family A receptors is the arginine in the Asp-Arg-Tyr (DRY) motif at the cytoplasmic side of transmembrane segment (TM) 3 (Probst et al., 1992) which is supposed be involved in G-protein activation. Nevertheless, six different groups of receptors have been further classified within this family of receptors with regard to their phylogeny.

Family B receptors include approximately 20 different receptors for a variety of peptide hormones and neuropeptides, such as vasoactive intestinal peptide (VIP), calcitonin, PTH, and glucagon. Except for the disulfide bridge connecting the first (ECL 1) and second extracellular loops (ECL 2), family B receptors do not contain any of the structural features characterizing family A receptors. Notably, the important DRY motif is absent in family B receptors. The most prominent characteristic of family B receptors is a large (approximately 100 residues) extracellular amino terminus containing several cysteines, presumably forming a network of disulfide bridges (Ulrich et al., 1998).

Family C receptors are characterized by an exceptionally long amino terminus (500–600 amino acids). This family includes the metabotropic glutamate, the γ-aminobutyric acid (GABA), the calcium, the vomeronasal, the mammalian pheromone receptors, and the recently identified putative taste receptors. Family C receptors have, like family A and B receptors, two putative disulfide-forming cysteines in ECL 1 and ECL 2, respectively, but otherwise they do not share any conserved residues with family A and B receptors. All these receptors possess a very large extracellular domain which shares a low but significant sequence similarity with bacterial periplasmic binding proteins (PBPs). Inside the bacteria, these proteins are involved in the transport of various types of molecules such as amino acids, ions, sugars or peptides. They are constituted of two lobes separated by a hinge region, and several studies including X-ray crystallography indicated that these two lobes closed like a Venus' flytrap upon binding of the ligand. The glutamate binding site has been proposed to be equivalent to the known amino acid binding site of PBPs; therefore, it is believed that the amino terminus of family C receptors contains the ligand-binding site (O'Hara et al., 1993; Conn and Pin, 1997).

Despite the A-F classification is widely accepted, recently, Fredriksson et al. (Fredriksson et al., 2003) performed the first phylogenetic study of the entire superfamily of GPCRs in a single mammalian genome (comprising about 2% of the genes in the human genome) and proposed a more accurate classification. Their analyses showed that there are five main families of human GPCRs and that within each family they share a common evolutionary origin:

glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin (the GRAFS classification, based on the initials of the family names). Three of these families, the rhodopsin (A), secretin (B), and glutamate (C) families, correspond to the A-F clan system (Attwood and Findlay, 1994), whereas the two other families, adhesion and frizzled, are not included in the clan system. Nevertheless, the rhodopsin receptors make up the largest family, divided into four main groups with 13 distinct branches.



Family A
Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)
Neurotensin, TRH receptors
Opsin receptors
Adenosine and Cannabinoid receptors
Nucleotide, oxytocin and somatostatin receptors
Melatonin receptors

Family B
Calcitonin receptors
PTH receptors
Glucagon, VIP and secretin receptors
Latrotoxin receptors

Family C
Metabotropic glutamate receptors
Metabotropic GABA receptors
Calcium receptors
Vemeronasal pheromone receptors
Taste receptors

Figure 3: GPCRs classification. GPCRs can be divided into three major subfamilies A, B and C. Highly conserved key residues are indicated in red circles.

GPCR INTERACTING PROTEINS

Many GPCRs contain sequence motifs that are known to direct protein-protein interactions and, therefore, have the theoretical capacity to interact with a wide range of other proteins. Such interactions might determine receptor properties, such as cellular compartmentalization or signal selection, and can promote as well protein scaffolding into complexes that integrate function.

The topology of GPCRs allows several potential faces for these interactions; the extracellular loops are short and, thus, extracellular interactions are likely to be dominated by the N-terminal sequences, which can be very extensive. On the intracellular face, because both the C-terminal tail and the third intracellular loop can be of considerable size, these are the regions on which attention has been focused (Figure 4).

The nature of such interactions varies according to their nature, ranging from transitory interactions (for example for signaling purposes) to more “stable” interactions. However, GPCR-protein assemblies should be considered as dynamic complexes that contribute to the intricate and finely tuned process of downstream signaling.

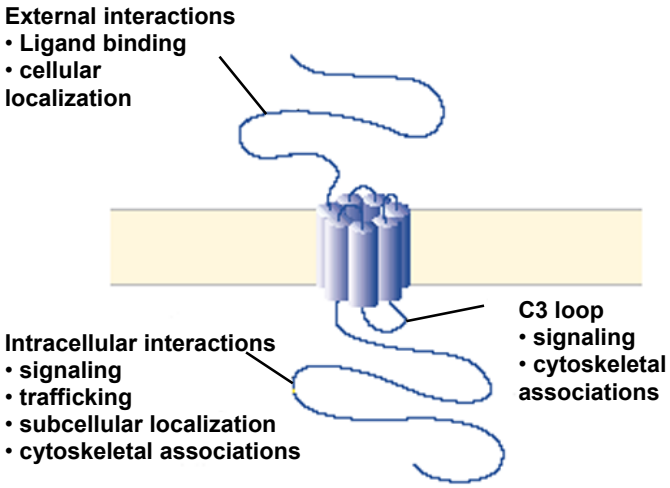


Figure 4: GPCR regions with identified interacting proteins. The generic functions of proteins that interact with these regions of GPCRs are indicated.

A large number of interactions with GPCRs in the intracellular side of the membrane have been described apart from those classically involved in signal transduction. An example of proteins interacting with GPCRs concerns to cytoskeletal associations since several GPCRs interact with cytoskeletal anchoring polypeptides. This is the case of α-filamin and dopamine D_2 receptors (Lin et al., 2001), α-actinin and adenosine A_{2A} receptors (Burgueno et al., 2003b) and the Shank family of proteins and several GPCR like type I metabotropic Glutamate receptor (mGluRI) or somatostatin receptor type 2 (SSTR2) (Sheng and Kim, 2000). In the last few years, interactions between GPCRs and PDZ-domain containing proteins have been reported. PDZ-domain containing proteins are known to play an important role in modulating signaling by defining the molecular composition of signaling complexes in a microcompartment and, in some cases, precisely localizing theses complexes within the cell. Thus for example, *NHERF* (Na^+/H^+ exchanger regulatory factor) interaction with β_2-adrenergic receptor has been shown to promote the clustering and the endocytosis of the receptor (Hall et al., 1998), and also, Homer-1b protein interaction with the mGluRI has been

demonstrated to modulate Ca^{2+} induced mobilization of these receptors (Roche et al., 1999). However, spynophilin (another PDZ-domain containing protein) has been found to interact with both, dopamine D_2 receptor and α_2-adrenergic receptor via a novel non-PDZ domain-mediated mechanism which allows it to act as a scaffold that links these GPCRs with signaling proteins like PP1 (Smith et al., 1999; Richman et al., 2001).

In addition to interactions at the intracellular faces of GPCRs, there is increasing evidence that extracellular protein-protein interactions might play roles in modulating the pharmacology of GPCRs or their subcellular localization. For example, the enzyme adenosine deaminase (ADA) a multifunctional protein which appears on the cell surface anchored to different proteins; in fact, our group obtained evidence that ADA could form heteromeric complexes with the adenosine A_1 and A_{2B} receptors (Saura et al., 1996; Herrera et al., 2001), interaction which seems to be essential for the high affinity agonist binding of A_1R.

G-PROTEIN COUPLED RECEPTOR-RECEPTOR INTERACTIONS

Apart from the interactions taking place in the intracellular and extracellular sides of the membrane, GPCRs are able to display protein-protein interactions at the membrane level with other receptors or ionic channels.

The widely recognized role of dimerization for many cell surface proteins contrasts with the classic models proposed for GPCRs, who were generally believed to function as monomers and to signal through downstream G-proteins in a 1:1 stoichiometric ratio.

Until recently, it was believed that distinct sets of intramolecular interactions within the receptor would characterize the active and inactive conformations upon binding of the ligands. But recent data indicate that in addition to the specific intramolecular interactions that could define the activation states of the receptor, intermolecular interactions might also be important. Receptor dimerization as well as interactions with accessory proteins have then been documented and proposed to participate in GPCR activity (Brady and Limbird, 2002).

Furthermore, during the last decade several findings have pointed out the fact that GPCR dimerization is not limited to homodimers (homomers), but that they can interact with both closely and distantly related members of the GPCR family to form heterodimers (hetero-oligomers). “Homodimerization” is defined as the physical association between identical proteins, whereas “heteromerization” is defined as the association between non-identical proteins. This association could be between two monomers to form dimers or between multiple monomers to form oligomers. As available techniques do not allow the distinction between dimers or higher-order oligomers, the term dimers is often used, being the simplest form of oligomer’s functional units that could explain the observations.

TECHNIQUES USED TO IDENTIFY GPCR DIMERS.

Pharmacological studies provided the first indirect evidence for the existence of GPCR dimers as complex radioligand-binding data demonstrating either positive or negative cooperativity hinted at the possibility of physical interactions between receptors (Mattera et al., 1985).

However, one of the first studies that renewed interest in the possibility that GPCRs could function as dimers was the elegant study by Maggio et al. (Maggio et al., 1993), using chimeric α_2-adrenergic/M3 muscarinic receptors composed of the first five transmembrane domains of one receptor and the last two transmembrane domains of the other. When either chimera was expressed alone, no binding or signaling could be detected, but coexpression of the two chimeras restored binding and signaling to both muscarinic and adrenergic ligands. Such functional trans-complementations were interpreted as intermolecular interactions between inactive receptors in a way that restored both ligand- binding and signaling domains within a dimeric complex.

Also consistent with this idea, several receptor mutants behave as dominant-negative mutants when expressed with their cognate wild-type receptor (Benkirane et al., 1997; Bai et al., 1998; Zhu and Wess, 1998). In these cases, dimerization between wild-type and the inactive receptor was invoked to explain the blunted response observed.

In recent years, one of the most common biochemical approaches used to investigate GPCR dimerization has been coimmunoprecipitation of differentially epitope-tagged receptors. The first study using such an approach was performed by Hebert et al (1996) demonstrating the specific interactions between β_2-adrenergic receptors (Hebert et al., 1996). Since then, similar strategies have been used to document homodimerization of the dopamine D_2 (Ng et al., 1996), the mGluR5 (Romano et al., 1996), the δ-opioid (Cvejic and Devi, 1997) and other receptors. More recently, coimmunoprecipitation experiments have also been used to demonstrate the existence of heterodimers between closely related receptor subtypes such as $GABA_BR1$ and $GABA_BR2$ (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) or δ- and κ-opioid receptors (Jordan and Devi, 1999), and even between more distantly related receptors as the adenosine A_1 and dopamine D_1 receptors (Gines et al., 2000), the angiotensin AT1 and bradykinin B2 receptors (AbdAlla et al., 2000) or the δ-opioid and β_2-adrenergic receptors (Jordan et al., 2001). However, although commonly used to study protein-protein interactions, coimmunoprecipitation of membrane receptors requires their solubilization using detergents, and it may be problematic when considering highly hydrophobic proteins such as GPCRs that could form artifactual aggregates upon incomplete solubilization, Despite all the controls used in to rule out this possibility, the general acceptance of GPCR dimerization still awaited the direct demonstration that these complexes existed in living cells. This was made possible with the development and utilization of biophysical methods based on light resonance energy transfer.

Light resonance energy transfer approaches are based on the non-radiative transfer of excitation energy between electromagnetic dipoles of an energy donor and acceptor. In the case of fluorescence resonance energy transfer (FRET), both the donor and the acceptor are fluorescent molecules, whereas for

bioluminescence resonance energy transfer (BRET), the donor is bioluminescent and the acceptor is fluorescent. A prerequisite for these processes is that the emission spectrum of the donor and the excitation spectrum of the acceptor must overlap and that the donor and the acceptor must be in close proximity (<100 Å).

BRET is a phenomenon occurring naturally in several marine animals such as de sea pansy *Renilla reniformis*. In this animal the luminescence resulting from the catalytic degradation of coelenterazine by luciferase (*Rluc*) is transferred to a variant of green fluorescent protein (GFP), which, in turn, emits fluorescence at its characteristic wavelength on dimerization of the two proteins. The strict dependence on the molecular proximity between donors and acceptors for energy transfer makes it a system of choice to monitor protein-protein interactions in living cells (Figure 5).

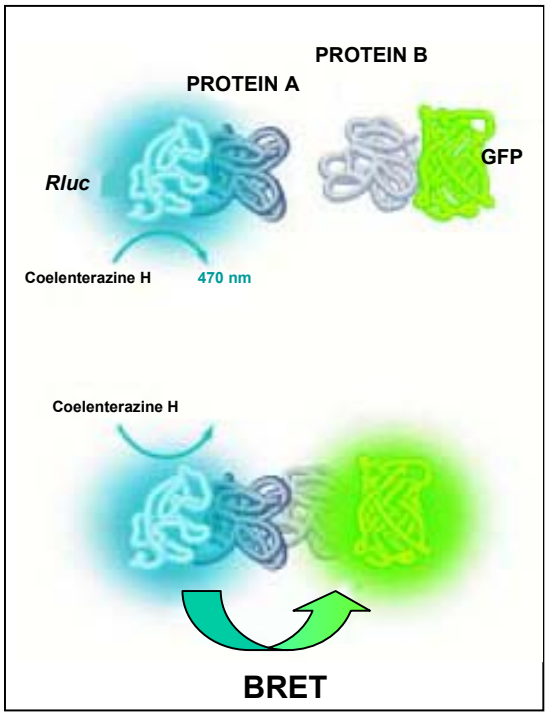


Figure 5. BRET Principle. Resonance energy transfer occurs when part of the energy (radiationless: dipole-dipole resonance) from Coelentrazine H (CH)-bound *Rluc* is transferred to GFP, which in turn, emits light. If *Rluc* and GFP are not in close proximity (upper panel), energy is not efficiently transferred and only the blue light emitted by the *Rluc*/CH reaction is detected. When *Rluc* and GFP are brought into close proximity (lower panel), by means of a specific biological interaction (Protein A and Protein B fused to *Rluc* and GFP, respectively), energy is efficiently transferred from CH-bound *Rluc* to GFP resulting in the production of yellow light from GFP. The BRET signal is measured by dividing the amount of yellow light by the amount of blue light.

During the last three years, research has taken advantage of this phenomenon to study dimerization of GPCRs. Fusion proteins that link GFP (or variants) and *Rluc* to the carboxyl terminus of individual GPCRs are constructed and coexpressed. In absence of dimerization, the addition of coelenterazine H

should lead to a characteristic broad bioluminescence signal with an emission peak at 470 nm, consistent with the spectral properties of *Rluc*. If dimerization occurs, the energy transfer between *Rluc* and GFP (resulting from the proximity between the bioluminescent and fluorescent fusion proteins) should lead to the appearance of an additional fluorescence signal with an emission peak at 530 nm that is characteristic of the GFP. Therefore, as a result of dimerization the ratio between emission at 530/emission at 470 increases (Figure 6).

However, since the efficiency of energy transfer decrease with the sixth potency of the distance between fluorophores, changes in BRET signal upon ligand treatment have to be interpreted with caution because the current approach doesn't allow the distinction between changes in the conformation of pre-existing dimers and an increase in their number.

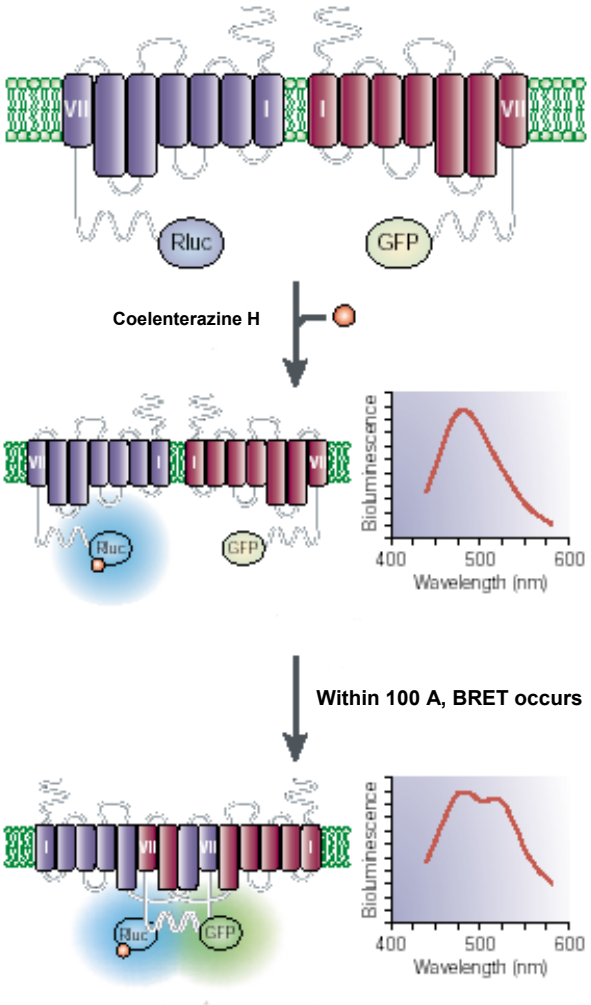


Figure 6: Schematic representation of BRET experiments carried out to detect GPCR dimerization.

Many of the studies characterizing dimerization have been carried out in heterologous expression systems, but a structural study performed by Palczewski et al., (Fotiadis et al., 2003) using atomic force microscopy, has demonstrated for the first time that rhodopsin exists as an array of dimers in the native retina, revealing an oligomeric organization of a GPCR *in vivo* (Figure 7).

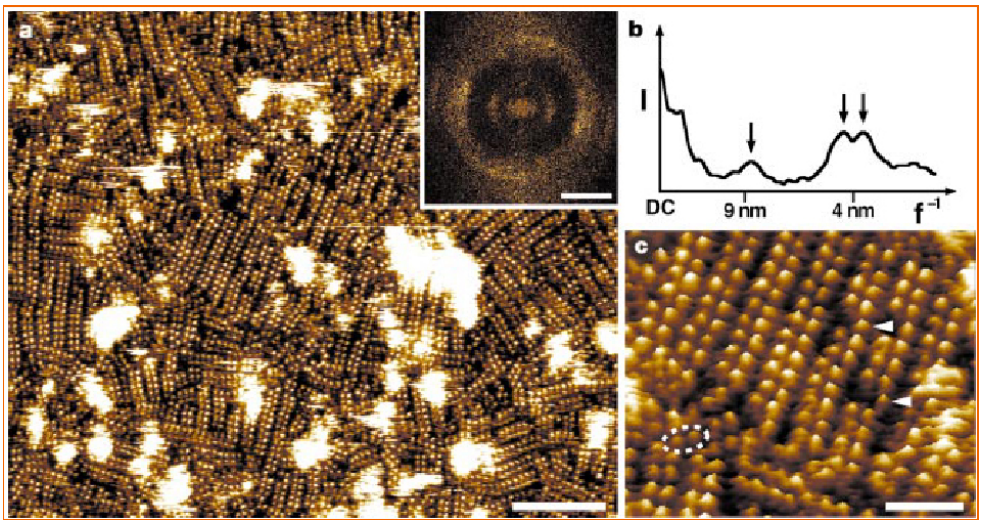


Figure 7: Organization and topography of the cytoplasmic surface of rhodopsin. **a,** Topograph obtained using atomic-force microscopy, showing the paracrystalline arrangement of rhodopsin dimers in the native disc membrane. Inset, arcs in the calculated powderdiffraction pattern reflect the regular arrangement of rhodopsin in the membrane. **b,** Angularly averaged powder-diffraction pattern. **c,** Magnification of a region of the topograph in **a**, showing rows of rhodopsin dimers, as well as individual dimers (inside dashed ellipse), presumably broken away from one of the rows, and occasional rhodopsin monomers (arrowheads), (Fotiadis et al., 2003).

FUNCTIONAL ROLE OF GPCR DIMERIZATION

The availability of a variety of techniques to study GPCR dimers has greatly facilitated studies to examine a role for dimerization in regulating receptor function. This regulation has been found to exist in different levels, from modulating receptor expression on the cell surface to conferring new pharmacological properties to the dimers. In addition, it has provided a new perspective to the signaling units of GPCRs as well as to the future development of drugs acting through these types of receptors.

One of the most significant observations to indicate that GPCR dimerization might be important in receptor folding and transport to the cell surface came from the studies of the metabotropic γ-aminobutiric acid receptor, $GABA_BR$. Three studies published simultaneously in 1998 (Jones et al., 1998; Kaupmann et al.,

1998; White et al., 1998) demonstrated that co-expression of two isoforms of the $GABA_B$ receptor, $GABA_BR1$ and $GABA_BR2$, was a prerequisite for the formation of a functional $GABA_B$ receptor at the cell surface. The analysis of this phenomenon showed that the isoform $GABA_BR1$ is retained intracellularly as an immature gylycoprotein and that, by contrast, the $GABA_BR2$ isoform is transported to the cell surface but cannot bind GABA or promote any intracellular signaling. In subsequent studies, Magreta-Mitrovick et al., demonstrated that $GABA_BR2$ serves as a chaperone that is essential for the proper folding and cell-surface transport of $GABA_BR1$. $GABA_BR1$- $GABA_BR2$ dimerization, through a coiled-coil interaction of the carboxyl tails, masks the ER-retention signal, thereby allowing ER transport and plasma membrane targeting of the dimer (Margeta-Mitrovic et al., 2000).

The role of dimerization as an early event in receptor maturation and transport is further supported by the observation that intracellular retention of dimers occurs when truncated forms of the V2 vasopressin and CCR5 chemokine receptors are expressed, causing nephrogenic diabetes and slow onset of AIDS respectively (Benkirane et al., 1997; Zhu and Wess, 1998).

Ligand-binding studies have provided some insight into the physiological relevance of GPCR homodimers and heterodimers since they can result in the generation of sites with novel ligand-binding properties. The first report about an heterodimer with distinct properties from their constituent receptors was with the heterodimer formed by κ- and δ-opioid receptors (Jordan and Devi, 1999). K-δ-receptor heterodimer do not exhibit high affinity binding for either κ- or δ- opioid receptor-selective dimers but, on the other hand, these heterodimers show high affinity for partially selective ligands. Studies with μ-δ-heterodimers also showed similar changes in the properties of binding sites.

Heteromerization of GPCRs not only results in changes in the affinity of the receptors for various ligands, it has also been shown that the binding of a specific ligand to one receptor in a heterodimer can alter the binding of a ligand specific for the neighbouring receptor, hinting at a possible mechanism where one ligand modulates the efficacy and/or potency of another ligand. Examples of this modulation are the heterodimer formed by somatostatin SSTR5 and dopamine D_2 receptors (Rocheville et al., 2000a) as well as the adenosine A_1-dopamine D_1 receptor heteromer, where A_1 agonists induced the disappearance of the high-affinity binding sites of the D_1 receptor in membrane preparations (Gines et al., 2000).

Some reports suggest that heteromerization can differentially affect the signaling by different agonists. One of the first evidence that dimers formed a complex signaling unit came form studies showing that the disruption of β_2-adrenergic dimers with a peptide derived from the 6^{th} transmembrane domain, inhibited agonist-induced cAMP production (Hebert et al., 1996). In addition, heteromerization between AT1 and bradykinin B2 receptors enhances the signaling of the AT1 receptor while inhibiting of the bradykinin B2 receptor, showing that heteromerization between different receptors may be a novel model for modulation of the response of GPCRs for their respective ligands (AbdAlla et al., 2000).

At this point it is noteworthy to mention that, since the receptors implied in the dimers can have different internalization characteristics, heteromerization can also modulate agonist-mediated trafficking properties of GPCRs. For example, this is the case of the somatostatin receptors SSTR1 and SSTR5 heterodimer, in which internalization occurs despite the internalization-resistance of the SSTR1 monomer (Rocheville et al., 2000b).

Table 1: Proposed functional roles for GPCR dimerization

Receptors	Evidence for function of dimerization
β2-Adrenergic	A peptide inhibiting dimerization, impairs agonist-stimulated adenylyl-cyclase activation.
Angiotensin	Coexpression rescues binding of 2 binding-deficient mutants.
V2 vasopressin	Dominant negative effect on WT receptor cell surface expression by truncated receptors.
Metabotropic Glutamate	Crystal structure of N-terminal domain is a constitutive dimer; ligand promotes a conformational change.
CCR5	Antibody-promoted dimerization inhibits HIV entry.
Somatostatin SST5-Dopamine D_2	Rescue of SST5 signaling impaired mutant by D_2R.
Somatostatin SSTR5-SSTR1	Functional complementation of binding-deficient mutant with signaling-deficient mutant; internalization of the dimer.
Angiotensin AT1-bradykinin B2	Increase efficacy and potency of angiotensin II but reduced bradykinin efficacy.
δ-κ opioid	Distinct pharmacological properties of heterodimers and synergistic activation of MAPK no effect of GTPγS on heterodimer.
δ-opioid - β2-Adrenergic	Crosstrafficking and synergistic activation of MAPK.
δ-μ opioid	Distinct pharmacological and signaling properties of heterodimers; no effect of GTPγS on heterodimer.
Muscarinin M3-M2	Distinct pharmacology of heterodimer
$GABA_BR1$-$GABA_BR2$	$GABA_BR2$ coexpression with $GABA_BR1$ necessary for cell surface expression of GABABR1 and normal GABA signaling; selective binding of a drug to a heterodimer subtype; allosteric cooperativity between R1 and R2 subtypes.
Adenosine A_1-Dopamine D_1	Desensitization of heterodimer upon pre-treatment with either agonist.
Dopamine D_2- D_3	Increased coupling of D_3 to adenylyl cyclase.
mGluR1- Adenosine A_1	Reciprocal enhancement of Ca^{2+} signaling.

ARCHITECTURE OF GPCR DIMERS

Distinct intermolecular interactions have been found to be involved for various GPCRs homo- and heteromers. Disulphide bonds have been found to be important for the dimerization of the calcium-sensing and metabotropic glutamate receptor (Romano et al., 1996; Bai et al., 1998), coiled-coil interactions involving the carboxyl tail of the $GABA_BR1$ and $GABA_BR2$ receptors is involved in the formation of their heterodimer (Margeta-Mitrovic et al., 2000) and, finally, for monoamine receptors such as the β_2-adrenergic receptor and dopamine receptors, interactions between transmembrane domains have been proposed to be involved in their homodimers (Hebert et al., 1996; Ng et al., 1996) . However, these proposed modes, rather than reflecting different strategies used by receptors of different classes, indicate that multiple sites of interaction are involved in the assembly and stabilization of receptor dimers.

Recently, computational studies performed by Gouldson et al. (Gouldson et al., 2000) porposed two alternative three-dimesional models that could describe GPCR dimers, both of them supporting the involvement of the 5^{th} and 6^{th} transmembrane helices in the dimerization interface, as well as an important role for the third intracellular loop (Figure 8). The first model is the domain-swapping model, in which each functional unit within the dimer is composed of the first five transmembrane domains of one polypeptide chain and the last two of the other. This model would rationalize the functional complementation observed when mutant or chimeric receptors were studied. The second model is the contact model, in which each polypeptide forms a receptor unit that touches the other through interactions involving transmembrane domains five and six.

Nevertheless, the validation of these models awaits additional studies, the most direct of which would come from the resolution of the structure of a GPCR receptor dimer.

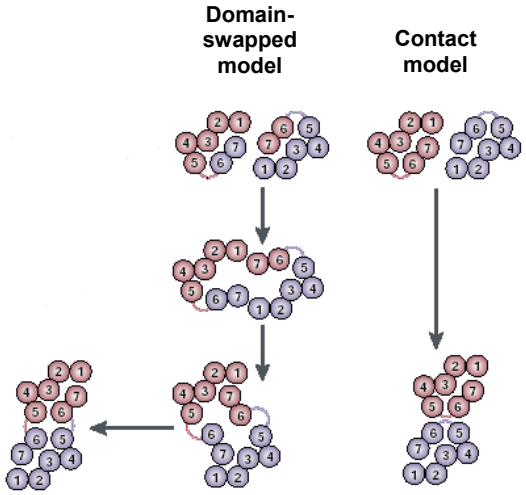


Figure 8: Alternative three-dimensional models showing dimers of GPCRs: domain swapped and contact model.

Oligomeric assembly of proteins, allowing expanded diversity with a limited number of modular elements, is the rule rather than the exception in biology. When considering the nervous system, the existence of homo- and heterodimers of neurotransmitter GPCRs raises the hypothesis that could underlie the high degree of diversity and plasticity that is characteristic of such a highly organized and complex system.

DOPAMINE RECEPTORS

STRUCTURE AND CLASSIFICATION

Dopamine constitutes about 80% of the catecholamine content in the brain.

Like other neurotransmitters, dopamine is not able of crossing the blood-brain barrier; however, the precursors of dopamine, phenylalanine and tyrosine are able to cross it. Thus, the biosynthesis of dopamine takes place within the nerve terminal's cytosol.

Release of dopamine into the synaptic cleft and the subsequent sequence of events follows the classic scheme of transmitter release: the calcium influx through voltage-dependent calcium channels triggers the fusion of the vesicle with the presynaptic membrane, a pore is formed from which dopamine is released into the synaptic cleft and dopamine diffuses across it and binds to postsynaptic receptors. The binding induces a change of conformation of the receptors, which in turn triggers the membrane permeability for ions and initiates a complex chain of intracellular postsynaptic events.

The outcome is an activation or inhibition of the postsynaptic neuron. Finally the dopaminergic signal is terminated by removal of dopamine from the intersynaptic cleft, which involves specific reuptake mechanisms into the presynaptic terminal where it can be stored and reused. Specific dopamine transporters (DAT) support the reuptake and play an important function in the inactivation and recycling of released dopamine.

Dopamine exerts its action by binding to specific membrane receptors which belong to the family of seven transmembrane domain (7TM) G-protein coupled receptors (Figure 9). In 1978, on the basis of pharmacological and biochemical evidence, dopamine receptors where first proposed to exist as two discrete populations: one positively coupled to adenylyl cyclase (AC) and the other one independent of the cAMP generating system (Spano et al., 1978). In 1979, Kebabian and Calne summarized these observations and suggested to call D_1 the receptors which stimulated AC and D_2 the ones that were not coupled to this effector (Kebabian and Calne, 1979). Subsequent studies confirmed this classification scheme, and D_1 and D_2 receptors were clearly differentiated pharmacologically, biochemically, physiologically, and by their anatomic distribution. Nevertheless, during the last decade, using gene cloning procedures, five distinct dopamine receptors have been isolated. These receptors have been classified into two subfamilies on the basis of their biochemical and

pharmacological properties: the D_1-like, which comprises D_1 and D_5Rs and the D_2-like, which includes D_2, D_3 and D_4Rs. As the first classification proposed, the D_1-like receptors are positively coupled to AC by G_S proteins while the D_2-like receptors inhibit this cyclase and activate K^+ channels as they are coupled to $G_{i/o}$ proteins (Dal Toso et al., 1989; Sunahara et al., 1990; Civelli et al., 1993).

The genomic organization of the dopamine receptors supports the concept that they derive from the divergence of two gene families that mainly differ in the absence or the presence of introns in their coding sequences. D_1 and D_5 do not contain introns in their coding regions (Gingrich and Caron, 1993), a characteristic shared with most G-protein coupled receptors (Dohlman et al., 1987). In contrast, and by analogy with the gene of rhodopsin, the genes encoding the D_2-like receptors are interrupted by introns; the coding region for D_2 contains six introns (Giros et al., 1989), the D_3 lacks the fourth intron of the D_2 and the D_4 lacks the third and the fourth (Bunzow et al., 1988). The presence of introns within the coding region of D_2-like receptors allows the generation of receptor variants. Indeed, the D_2 receptor has two main variants, called D_{2S} and D_{2L}, which are generated by alternative splicing of an 87-bp exon between introns 4 and 5.

All of the dopamine receptors subtypes share several conserved residues with in their TMD, which are thought to be the minimal requirements for catecholamine binding: the two serine residues in the fifth TMD are thought to be involved in recognition of the two hydroxyl groups of catecholamines and the aspartic acid residue in the third TMD is thought to act as a counterion for the amine group in biogenic amines (Hibert et al., 1991).

Post-translational modifications of these receptors differ depending on the subtype. D_1-like receptors have two consensus sites for N-linked glycosylation: in the N-terminal domain and in the second extracellular loop. On the other hand, for the D_2-like subtype, D_2 and D_3Rs can be multiply glycosylated in four potential sites of their extracellular loops while D_4R has only one potential site for N-linked glycosylation. On the contrary, the C-terminus in both subfamilies contains phosphorylation and palmitoylation sites which are thought to be involved in agonist-dependent receptor desensitization and in the formation of the fourth intracellular loop (Figure 9). Finally, it is widely believed that the intracellular domains of the 7TM receptors are responsible for the specificity and coupling of these receptors to G-proteins.

Despite the pharmacological properties of dopamine receptors has still to be determined in living animals, dopaminergic ligands easily discriminate between the D_1- and D_2-like receptor subfamilies. However, most of them do not clearly differentiate between members of the same subfamily.

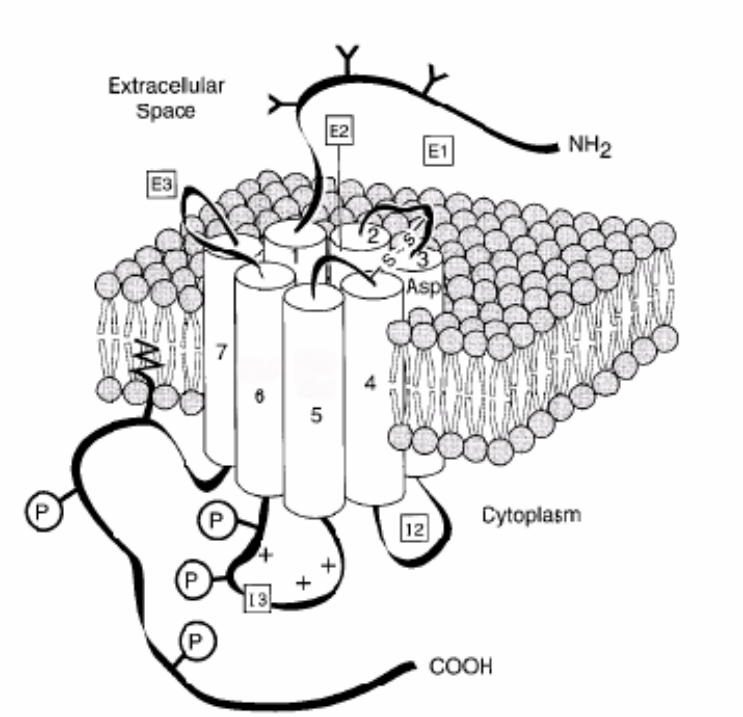


Figure 9: Dopamine receptor structure. Residues involved in dopamine binding are highlighted in transmembrane domains. Potential phosphorylation sites are represented on 3rd intracellular loop (I3) and on COOH terminus. Potential glycosylation sites are represented on NH2 terminal. E1-E3, extracellular loops; 1–7, transmembrane domains; I2-I3, intracellular loops.

The D_1-like receptors share high homology within their TM domains, the regions which are thought to form the ligand-binding site. Thus, it is not surprising that these receptors exhibit very similar ligand binding properties (See Table 2). Each of these receptors exhibits the classical ligand binding characteristics of D_1 receptors: high affinity for benzazepines (SCH-23390 and SKF-38393) and low affinity for butyrophenones (spiperone and haloperidol) and substituted benzamides (sulpiride). An interesting difference between the D_1-like receptors is their relative affinity for dopamine, as the human D_5R has been reported to have an affinity ten-fold higher for dopamine that the D_1R. The only antagonist shown to have different affinities for these receptors is spiperone, ten-fold less potent at D_5 than D_1R.

The pharmacological properties of the D_2-like receptors differ more than has been observed for D_1-like receptors. As seen in Table 2, affinities for several antagonists and agonists vary one to two orders of magnitude between subtypes. Each of these receptors, however, has the hallmark ligand-binding characteristics of D_2-like receptors which are high affinity for such butyrophenones as spiperone and haloperidol, and low affinity for such benzazepines as SKF-38393. Of the three D_2-like receptors, the D_4R has the most distinctive pharmacological properties. In general, D_4R displays lower affinities for most dopaminergic antagonists; for example, D_2 and D_3Rs have a relatively high affinity for such benzamides as raclopride, whereas D_4R has a very low affinity for this compound. However, the D_4R exhibits relatively high affinity for the atypical neuroleptic clozapine.

Table 2: Pharmacological properties of dopamine receptors Values for K_D in nM.

	D1-like		D2-like		
	D1	**D5**	**D2**	**D3**	**D4**
Agonists					
Quinpirole	1900		4.8-576	5.1-24	30-46
Dopamine	0.9-2340	<0.9-261	2.8-474	4-27	28-450
(-)Apomorphine	0.7-680	122-163	0.7-24	20-32	4
Bromocriptine	440-672	450	5.3-12.6	5-7.4	290-340
7-OH-DPAT	5000		10-103	1-2	650
cis-8-OH-PBZI	21230	15060	2470	27	276
SKF-38393	1-150	0.5-100	150-9560	5000	1000-1800
NPA	421	187	0.4-0.9		6.5
Antagonists					
AJ-76			80-270	35-91	
(+)Butaclamol	0.9-3	5-27	0.69-0.8	4.1-11.2	38-51
Clozapine	100-261	194-336	56-230	83-620	9-42
Chlorpromazine	73-90	130-314	0.5-3	1.16-6.1	35
Domperidone			0.3	9.5	
Haloperidol	27-203	33-151	0.6-1.2	2.74-7.8	2.3-5.1
Spiperone	99-350	135-4500	0.06-0.37	0.32-0.71	0.05-4
Raclopride	18000		1-5	1.8-3.5	237-2400
Remoxipride			54-300	969-1600	2800-3690
Risperidone			1.7-5	6.7	7
SCH-23390	0.11-0.35	0.11-0.54	270-1100	314-800	3000-3650
S(-)Sulpiride	20400-45000	11000-77270	2.5-71	8-206	21-1000
(+)S-14297			297	13	
U-99194A			1572	78	
UH-232			13-40	2.9-9.2	

DOPAMINE D_2 RECEPTORS

The D_2 subtype of dopamine receptors represents the main presynaptic autoreceptor of the dopaminergic system (Mercuri et al., 1997), but it is also critical for postsynaptic transmission (Rouge-Pont et al., 2002). This receptor has been the subject of extensive studies, which have demonstrated its participation in numerous important physiological functions, such as synthesis and release of pituitary hormones and control of motor activity. Dopamine D_2Rs represent the major target of antipsychotic drugs and are involved in various neuropathological conditions including Parkinson's disease, Tourette's syndrome and drug addiction (Vallone et al., 2000).

The rat D_2R was the first dopamine receptor cDNA to be isolated (Bunzow et al., 1988). This original cDNA contains an open reading frame of 1245 nucleotides encoding a protein of 415 residues, later called D_{2S} (D_2 short), with a typical D_2 pharmacological profile. Later, several groups cloned a splice variant of this receptor, D_{2L} (D_2 long), from different species (rat, mouse, bovine, human) and tissues (brain, pituitary and retina). The D_2R gene is composed of eight exons, seven of which are coding. The alternative splicing of the sixth exon, which encodes for a 29 amino acid insertion in the third intracellular loop, generates the D_2L and D_2S isoforms.

The D_2R is mostly found in brain tissues, such as the caudate-putamen, olfactory tubercle and nucleus accumbens, where it is expressed by GABAergic neurons coexpressing enkephalins. In addition, the mRNA of this receptor is also found in the substantia nigra and in the ventral tegmental area, the nuclei that give

rise to the major dopaminergic tracts of the brain, which indicates that the D_2R is one of the main dopamine receptors that directly control the activity of dopamine-containing neurons. However, the D_2R is also found outside the central nervous system, in the anterior and neurointermediate lobes of the pituitary gland, which indicates that it is one of the primary dopamine receptors that regulate hormone release (Vallone et al., 2000).

In general, there appears to be little tissue specificity to the expression of the long and short forms of the D_2R. However, studies performed by using dopamine $D_{2L}R$-null mice indicate a preferential involvement of D_2L receptors in postsynaptic responses while the $D_{2S}R$ seems to be preferentially expressed by midbrain dopaminergic neurons where it acts as an inhibitory autoreceptor (Lindgren et al., 2003).

Previous studies have shown that D_{2L} and D_{2S} receptors bind to distinct G-proteins, most likely as a result of their structural differences. However, both isoforms function by binding to the pertussis toxin-sensitive G-proteins G_i or G_z, both of which have an inhibitory effect on adenylyl cyclase that seems to be the predominant signaling pathway utilized by D_2Rs in the central nervous system.The D_2R G-protein coupling has also been described to modulate the activation of K^+ currents leading to cell hyperpolarization (Missale et al., 1998).

Suppression of Ca^{2+} currents through L-type voltage dependent Ca^{2+} channels (L-type VDCC) is one of the best established adenylyl cyclase-independent signaling pathways of D_2 receptors demonstrated in cell lines, lactotroph cells and striatal neurons (Ghahremani et al., 1999; Banihashemi and Albert, 2002). This pathway seems to involve the βγ subunits of the G-protein, PLC activation, IP3 dependent-intracellular Ca^{2+} mobilization and activation of the Ca^{2+}-dependent serine-threonine phosphatase calcineurin (PP-2A) (Hernandez-Lopez et al., 2000). In addition, calcineurin has also been shown to be a main phosphatase involved in dephosphorylation of DARPP-32. Therefore, D_2 receptor activation produces DARPP-32 dephosphorylation both by inhibiting adenlilate cyclase activity and by a Ca^{2+}/calcineurin-dependent and adenylyl cyclase-independent mechanism (Nishi et al., 1997).

Finally, dopamine D_2Rs stimulation has also been described to induce mitogen-activated protein kinase (MAPK) and cAMP response element-binding protein (CREB) phosporylation in neurons (Yan et al., 1999). CaMK, PKC, DARPP-32 and elevated Ca^{2+} seem to be important in this pathway. Activation of MAPK has been proposed to play an important role in dopamine-induced regulation of gene expression and long term neuronal adaptation in the striatum.

Baik et al generated the D_2R knockout mice in 1995 (Baik et al., 1995). These mice showed a striking impairment of motor behaviour (parkinsonian-like phenotype) supporting an essential role for these receptors in the dopaminergic control of physiological function connected to movement. However, dopaminergic neurotransmission is not the only system involved in the control of motor function in the striatum. Another key regulator of basal ganglia activity is adenosine, that acting through its specific receptors has been shown to have numerous effects on neurotransmitter release in the striatum and, in particular, in the striatopallidal pathway.

ADENOSINE RECEPTORS

STRUCTURE AND CLASSIFICATION

Adenosine and its derivatives are essential constituents of all living cells as a well-known building blocks for many biologically relevant molecules like ATP, NAD^+, SAM or nucleic acids.

The fact that, under normal conditions, adenosine is continuously formed intracellularly as well as extracellularly, suggests that basal concentrations of this nucleoside probably reflect an equilibrium between its synthesis and its degradation processes. However, when adenosine levels in the extracellular space are high, adenosine is transported into cells by means of specific transporters. It is then phosphorylated to AMP by adenosine kinase or degraded to inosine by adenosine deaminase. Adenosine deaminase, but not adenosine kinase, is also present in the extracellular space, what makes it another regulator of the extracellular concentration of adenosine (Franco et al., 1998).

Despite their role in cellular metabolism, extracellular purines and purine nucleotides (adenosine, ADP and ATP) exert a regulatory function by acting through specific membrane receptors that mediate both, normal and pathophysiological processes, the purinergic receptors. Purinergic receptors are classified into two main classes: P1 receptors (or adenosine receptors) and P2 receptors (or nucleotide receptors) (Fredholm et al., 1994). The P1 receptors are G-protein coupled receptors which have adenosine as their natural ligand. P2 receptors can bind a wide variety of ligands (ATP, ADP, UTP, UDP and Ap_nA) and have been classified by their molecular structure and signal transduction mechanisms into two subtypes, the P2X type are ionic channels which mainly bind ATP while P2Y receptors are G-protein coupled receptors (Abbracchio and Burnstock, 1994).

Adenosine receptors have been classified on the basis of their molecular, biochemical and pharmacological properties into four subtypes, A_1, A_{2A}, A_{2B} and A_3.

The amino acid sequence of all the cloned adenosine receptors is consistent with the three-dimensional structure proposed for the G-protein coupled receptors (Hibert 1991). According to this model these receptors consist of seven alpha-helical membrane-spanning domains connected by three extra- and three intracellular loops and the N- and C- termini of the protein on the extra- and intracellular sides of the plasma membrane respectively. The highest homology between subtypes is located in the transmembrane regions. These regions, together with the second extracellular loop, are supposed to be involved in ligand binding and recognition (Rivkees et al., 1999), while the G-protein interaction is supposed to occur basically in the third intracellular loop.

Moreover, adenosine receptors contain several features common to all G-protein coupled receptors which display this structure (Figure 10):

- The presence of Cys residues on the extracellular loop which may be involved in disulfide bond formation which would confer a conformational stability to receptors after insertion to the plasma membrane (Dohlman et al., 1990).
- All the cloned adenosine receptors present a “DRY” sequence which has been suggested to mediate G-protein activation.
- Each of the adenosine receptors possesses consensus sites for N-linked glycosilation on their second extracellular loops. Although it is supposed to be implied in membrane targeting for the receptor, the role of glycosilation is still not clear (Klotz and Lohse, 1986; Stiles, 1986).
- A_1, A_{2B} and A_3, but not A_{2A} have a Cys residue in their C-terminal domain which can be palmitoylated. This modification would allow another insertion in the membrane generating a fourth intracellular loop that has been suggested to participate in the coupling of the receptor to the G-protein. (Bouvier et al., 1995).

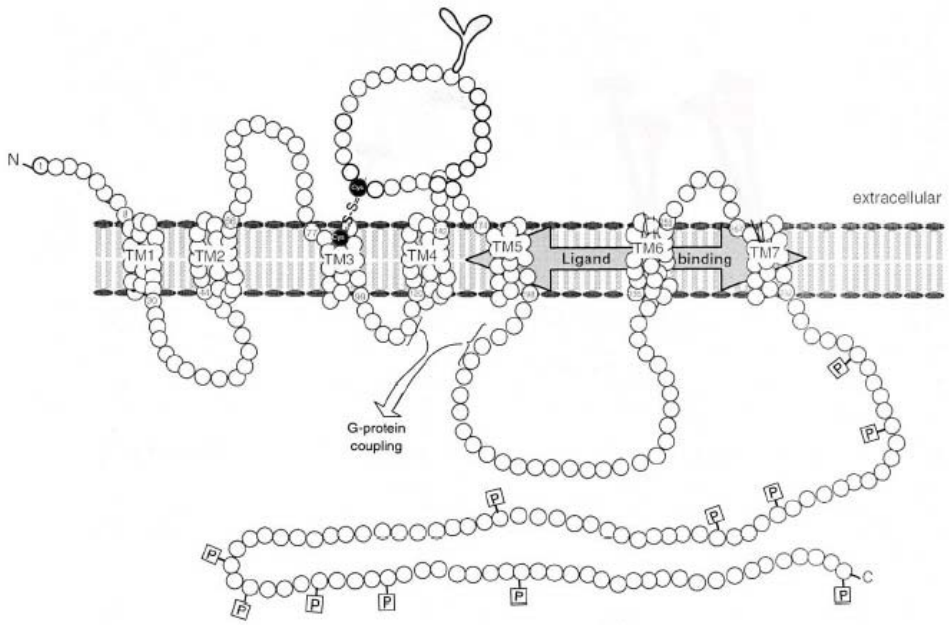


Figure 10: Structural model of adenosine receptors. The critical regions and residues possibly involved in ligand binding,G-protein coupling, glycosylation and phosphorilation are indicated.

The adenosine A_1 and A_{2A} receptors were initially subdivided on the basis of their inhibiting and stimulating adenylyl cyclase respectively (Fredholm et al., 2001). Indeed, A_1 and A_3 receptors are mainly coupled to the G_i protein subtype (although G_o and G_q have been described respectively) while the A_{2A} receptor can couple to G_s or to the G_{olf} subtype. Depending on the subtype, the activation of the G-protein gives rise to different signaling events. Thus, A_1 receptors mediate inhibition of adenylyl cyclase, activation of several types of K^+-channels (probably via βγ subunits), inactivation of N-, P-, and Q-type Ca^{2+}-channels and activation of phospholipase Cβ. A_{2A} and A_{2B} receptor-mediated stimulation of the formation of cAMP seems to be the main signaling event for these receptors. Nevertheless, coupling of the A_{2B} receptor with G_q proteins mediates PLC activation and, then, mobilization of intracellular calcium (Table 3). Finally, all adenosine receptors have shown a positive coupling to the MAPK cascade but, depending on the cellular background, the required signaling elements vary widely (Fredholm et al., 2001).

The endogenous agonist and natural ligand adenosine is of limited interest as a tool for the investigation of adenosine receptors due to its susceptibility to extensive metabolism by several enzymes. Nevertheless, adenosine is the central core of all agonists known to date (Table 3). Three positions in the molecule may be modified to increase affinity to specific receptor subtypes without destroying the agonistic activity: the 5'-position of the ribose and the 2- and N^6 positions of the purine. Methylxantines constitute the prototypical group of antagonists and modifications to this molecules result in a huge selection of derivatives some of which show distinct subtype selectivity. More recently, other structures including triazoloquinazolines, triazolotriazines, dihydropyridines and adenine derivatives served as the basis for a variety of non-xanthine antagonists. It is important to notice that there is no selective ligand for the A_{2B} receptor; NECA as agonist and XAC and CGS15943 as antagonists are being used despite that they display higher affinity for A_1 or A_{2A} receptors.

PHYSIOLOGICAL ROLES OF ADENOSINE IN THE BRAIN

Adenosine, acting through different receptor subtypes, plays important roles in the modulation of many cell functions which are summarized in Table 4.

The generation of genetically modified mice during the last few years has confirmed some of these functions and provided new insights into the physiology and pathophysiology of the different adenosine receptors. The knockout mice of A_{2A} receptor showed that these receptors play a role in mediating pain via peripherial sites, inhibiting platelet aggregation and regulating blood pressure (Ledent et al., 1997); mice with a targeted disruption of the A_3 receptor show a decreased effect of adenosine analogs on mast cell degranulation (Salvatore et al., 2000) with a consequent decrease in vascular permeability (Tilley et al., 2000) and, finally, A_1R knockout mice show increased anxiety, hyperalgesia, total

elimination of adenosine effects on excitatory neurotransmission and the neuronal response to hypoxia is markedly altered (Johansson et al., 2001).

Table 3: Signaling, pharmacology and distribution of adenosine receptors

Receptor subtype	A_1	A_{2A}	A_{2B}	A_3
G-protein	$G_{i/o}$	G_s/G_{olf}	G_s/G_q	Gi/G_q
Effects of G-protein coupling	⇩AMPc ⇧IP_3 ⇧K^+ ⇩Ca^{2+}	⇧AMPc	⇧AMPc ⇧IP_3 ⇧Ca^{2+}	⇩AMPc ⇧IP_3
Selective agonists	CPA CCPA CHA R-PIA	CGS21680 HE-NECA APEC CV 1808 DPMA WRC-0740	-	IB-MECA 2Cl-IB-MECA
Selective antagonists	DPCPX XAC KW-3902 ENX KFM 19 N 0861 FK 453 WRC 0571	KF17837 ZM241385 CSC SCH 58261	-	L-268605 L-249313 MRS 1067 MRS 1097
High Expression tissues	Brain (cortex, cerebellum, hippocampus); dorsal horn of spinal cord; eye; adrenal gland and atria	Spleen; thymus; leucocytes; blood platelets; striatopallidal GABAergic neurons (in caudate-putamen, nucleus accumbens and olfactory tubercle)	Caecum; colon and bladder	Testis and mast cells

Adenosine regulates many physiological processes, particularly in excitable tissues such as heart and brain. Many of the actions of adenosine either reduce the activity of excitable tissues (e.g. by slowing the heart rate) or increase the delivery of metabolic substrates (e.g. by inducing vasodilation) and, thus, help to couple the rate of energy expenditure to the energy supply. However, this type of unitary role for adenosine is not sufficient to explain many of its actions, and it is clear that adenosine plays a variety of different roles as an intercellular messenger. This is particularly the case in the brain, which expresses high concentrations of adenosine receptors, and where adenosine has been shown to be involved in both normal and pathophysiological processes, including regulation of sleep, arousal, neuroprotection, and epilepsy.

The idea that adenosine plays a role in sleep is a natural outgrowth of the observation that adenosine receptor antagonists such as caffeine promote wakefulness and disrupt normal sleep. Although many studies have implicated A_1 receptors in both sleep and decreased arousal (Fulga and Stone, 1998), there is also evidence that A_{2A} receptors may be involved, particularly in the rostral basal forebrain, where the A_{2A} agonist CGS21680 promotes both REM (rapid eye movement) and non-REM sleep (Satoh et al., 1999).

It has long been recognized that adenosine is involved in the autoregulation of cerebral blood flow (Wahl and Schilling, 1993), where it modulates vascular resistance via A_{2A} receptors (Coney and Marshall, 1998). Adenosine applied externally to cerebral blood vessels induces vasodilation, and there is evidence that endogenous adenosine is a tonic regulator of vascular smooth muscle tone. Thus, application of adenosine antagonists causes vasoconstriction and reverses adenosine-mediated vasodilation (Ko et al., 1990). Accordingly, any stimulus that promotes release of additional adenosine from neurons or glia will induce vasodilation. It has been suggested that this relationship between adenosine and cerebral blood flow is a mechanism that couples increased cell energy expenditure (seen as increased ATP utilization and demand) with increased oxygen and glucose delivery via the cerebral vasculature. The increased adenosine released during such conditions as ischemia would serve to increase cerebral blood flow and could ameliorate the effects of ischemia.

Extracellular brain concentrations of adenosine are markedly elevated by a diverse array of pathological stimuli. Many of the effects of adenosine that are observed to a minor extent under normal conditions are greatly augmented during pathological events and are neuroprotective in that context. The neuroprotective actions of adenosine are mediated primarily via A_1 receptor activation, and at least three cellular mechanisms may be involved. Adenosine strongly inhibits transmitter release (and glutamate in particular), hyperpolarizes neurons, and directly inhibits certain kinds of Ca^{2+} channels. All these actions could reduce excitotoxicity by limiting Ca^{2+} entry, which is thought to be a key step in excitotoxic damage, and by reducing metabolic demand, which would help to preserve ATP stores that are essential for pumping Ca^{2+} out of the cell. Alternatively, some of the protective effects could be mediated by other receptors (e.g. the A_3 receptor), which has a substantially lower affinity for adenosine and thus, would require higher concentrations for maximal activation. The A_{2A} receptor, on the other hand, may

actually contribute to ischemic tissue damage, because mice lacking A_{2A} receptors show reduced brain damage following focal ischemia (Chen et al., 1999).

In addition to having acute protective effects, transient activation of adenosine receptors offers protection against damage induced by a subsequent hypoxic or ischemic event. This phenomenon, which is referred to as preconditioning, occurs not only in brain but also in other excitable tissues, such as heart (Miura and Tsuchida, 1999). This effect seems to involve A_3 as well as A_1 receptors. In the brain, adenosine release, A_1 receptor activation, and the opening of ATP-dependent K^+channels appear to play a central role in preconditioning. Much clinical interest is focused on determining how to maximize acute neuroprotection, and how to take advantage of the preconditioning phenomenon in both the brain and the heart to improve patient outcome (Liang and Jacobson, 1999).

Finally, adenosine has also been demonstrated to be involved in other pathological states such as epilepsy, where it acts an anticonvulsant in experimental models, or in the actions of drugs of abuse, where adenosine signalling pathways may offer new targets for the treatment of addiction (Manzoni et al., 1998).

The two major subtypes of adenosine receptors in the brain are A_1 and A_{2A} which are expressed pre- and postsynaptically. However, while A_1 are highly expressed in the whole brain, the expression of A_{2A} is mainly restricted to the striatum and other nuclei of the basal ganglia.

At the synaptic level, although adenosine is not a neurotransmitter on its own, it shares via A_1 activation many properties attributed to the major inhibitory neurotransmitter γ-aminobutiric acid (GABA). Thus, GABA and adenosine constitute key molecules in the control of glutamatergic synaptic transmission in the central nervous system. Adenosine is able to functionally disconnect GABAergic interneurones by inhibiting their glutamatergic input, a process that might be particularly relevant under conditions of intense adenosine release, such as hypoxia (Sebastiao et al., 2001). Thus, although indirectly, adenosine inhibitory A_1 receptors might control GABAergic functioning, direct excitation of GABAergic nerve terminals by adenosine might occur via A_{2A} receptors. Indeed the adenosine A_{2A} receptor system appears to be one of the first presynaptic neuromodulatory systems able to enhance the evoked release of GABA from hipocampal nerve terminals (Cunha and Ribeiro, 2000).

Finally, there are some evidences which indicate that adenosine, acting through A_1 and A_{2A}Rs, may influence the developing nervous system. In addition, the neuroprotective role of adenosine under conditions of ischemia, it has been reported that both receptors are expressed during late prenatal periods and that their levels increase after birth. The expression pattern of A_1Rs in brain during the second half of gestation leads the possibility that the activation of these receptors may influence the differentiation and migration of neurons, which takes place at this period of neural development (Rivkees, 1995). Similarly, A_{2A}Rs expression in brain has been reported to occur during development, which suggests a role of these receptors in neuronal differentiation and synaptogenesis (Svenningsson et al., 1999).

TABLE 4. Physiological responses to adenosine and receptor subtypes involved.

Physiological response	Receptor subtype
Central nervous system	
Sedation	A_1
Decrease of locomotor activity	A_{2A}
Anticonvulsant action	A_1
Inhibition of neurotransmitter release	A_1
Peripherial nervous system	
Inhibition of sympathetic nerves	A_1
Inhibition of parasympathetic nerves	A_1
Stimulation of chemoreceptor	A_{2A}/A_{2B}
Stimulation of parasympathetic nerves	A_{2A}
Modulation of sensory nerves	$A_1/ A_{2A}/A_{2B}/A_3$
Cardiovascular system	
Vasodilation (vascular smooth muscle)	$A_{2A}/A_{2B}/A_3$
Vasoconstriction	A_1
Preconditioning	A_1/A_3
Inhibition of platelet aggregation	A_{2A}
Respiratory and pulmonary system	
Bronchodilation	A_{2A}/A_{2B}
Bronchoconstriction	A_1
Renal system	
Antidiuresis (tubular sodium reabsortion)	A_1
Constriction of afferent arteriole	A_1
Dilation of efferent arteriole	A_{2A}
Contraction of mesangial cell	A_1
Tubuloglomerular feedback response	A_1
Inhibition/stimulation of renin release	A_1/A_{2A}
Gastrointestinal system	
Inhibition of gastric acid output	A_1
Stimulation of intestinal secretion	A_{2A}
Hormonal and exocrine system	
Inhibition of lipolysis	A_1
Increase of insulin sensitivity	A_1
Inhibition of pancreatic insulin secretion	A_1
Stimulation of pancreatic glucagon secretion	A_{2A}/A_{2B}
Stimulation of gluconeogenesis	A_{2A}/A_{2B}
Immune system	
Inhibition of neutrophil superoxide generation	A_{2A}
Inhibition of expression of cell adhesion molecules	A_{2A}
Inhibition of cytokine synthesis	$A_{2A}/A_{2B}/A_3$
Degranulation of mast cell	A_{2A}/A_{2B}
Promotion of neutrophil neurotaxis	A_{2A}/A_{2B}
Inhibition of eosinophil migration	A_3
Immunosupression	A_{2A}

ADENOSINE A_{2A} RECEPTORS

The human A_{2A} receptor gene is located at chromosome 22q11.2 and contains 2 exons interrupted by a single intron of nearly 7 kb between the regions encoding transmembrane domains III and IV (Peterfreund et al., 1996). Two clusters of alternative transcripts exist as shown by primer extension and RNase protection analysis with PC12 cell RNA. Consistent with two clusters of transcription start sites, two independent functional promoter regions have been identified what suggests a potentially different usage of the two promoters in different cell types (Stehle et al., 1992).

Regarding to the A_{2A} receptor distribution, in the brain, by in situ hybridization its mRNA at first seems restricted to the striatum, nucleus accumbens and olfactory tubercle. However, using more sensible techniques, distinct levels of this receptor are also readily found in the cortex, amygdala, olfactory tubercles, hippocampus, hypothalamus, thalamus and cerebellum. In the periferial tissues, adenosine A_{2A} receptor is also expressed in spleen, thymus, heart, lung, kidney, leucocytes and in blood platelets (Moreau and Huber, 1999).

The major signal transduction pathway used by A_{2A} receptors depends on the activation of adenylyl cyclase, by means of G_s/G_{olf} coupling (Kull et al., 2000). A_{2A} receptor mediated adenylyl cyclase activation generates cAMP, which activates a cAMP-dependent protein kinase (PKA), which in turn regulates the state of phosphorylation of various substrate proteins. One of these substrates is DARPP-32, which is phosphorylated by PKA at a single site that converts this protein into a potent inhibitor of protein phosphatase-1 (PP-1), a phosphatase that inhibits CREB activity (Nishi et al., 2000). In this line of evidence, it has been demonstrated that, through PKA activation $A_{2A}R$ stimulation in the GABAergic striatopallidal neurons can potentially produce a sustained increase in the transcription of some CREB modulated genes by a mechanism involving increased CREB phosphorylation and decreased CREB dephosphorylation. The immediate-early gene *c-fos* and the preproenkephalin and neurotensin genes are very well studied target genes the promoters of which contain consensus sites for CREB-P binding (Borsook and Hyman, 1995). Recent studies have shown that A_{2A} receptor stimulation can, under certain conditions, increase the expression of *c-fos*, preproenkephalin and neurotensin genes. In addition to the preferential $G_{s(olf)}$/adenylyl cyclase pathway, A_{2A} receptors have also been shown to use other signaling pathways, such as PLC (Wirkner et al., 2000), phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK (Seidel et al., 1999; Schulte and Fredholm, 2003).

To gain a deeper understanding of the putative functions of the A_{2A} receptors *in vivo,* A_{2A} receptor-deficient mice have been generated (Ledent et al., 1997). These homozygous mice are viable and develop normally, suggesting that the A_{2A} receptor function may not be critical during neurogenesis. However, these A_{2A} receptor deficient mice display behaviours reflecting increased anxiety and hyperagression (in males). The neural basis of these behaviours is uncertain because developmental compensatory mechanisms involving other adenosine receptors are possible.

EVIDENCE OF INTERACTIONS BETWEEN ADENOSINE AND DOPAMINE RECEPTORS.

In the last decade, the accumulation of many experimental findings indicates that dopaminergic neurotransmission is involved in the motor effects of adenosine agonists and antagonists. This was first suspected with the observation that the motor activation induced by adenosine antagonists was counteracted by treatments that caused an acute dopamine depletion or dopamine receptor blockade. Furthermore adenosine receptor agonists inhibited and adenosine antagonists potentiated the motor activating effects of dopamine receptor agonists (Ferre and Fuxe, 1992). Therefore, an antagonistic interaction between adenosine and dopamine seemed to exist in the striatum, the main input structure of the basal ganglia and a key component of the motor system.

Even though adenosine A_1 receptors were suggested to modulate dopamine release in nerve terminals, most experimental findings could only be explained by an adenosine-dopamine interaction which took place postsynaptically.

Morophological and functional data supported the hypothesis of A_1-D_1 and A_{2A}-D_2 interactions:

- The existence of segregation of the different dopamine and adenosine receptor subtypes in the two different types of striatal GABAergic neurons. In the dorsal striatum dopamine D_1 receptors were mostly localized in the strionigral and strioentopeduncular neurons, while dopamine D_2 receptors were found in the striatopallidal neurons; similarly, adenosine A_{2A} receptors were almost exclusively restricted to the striatopallidal neurons, while adenosine A_1 receptors were found in both types of GABAergic neurons. These morphological observations suggested that striopallidal and strionigral-strioentopeduncular neurons might be the main locus for the A_{2A}-D_2 and A_1-D_1 interactions respectively (Ferre et al., 1996a).
- By using in vivo microdialysis, dopamine D_2 receptor agonist-induced decrease in extracellular GABA levels was shown to be completely counteracted by the adenosine A_{2A} agonist CGS21680 (thus mimicking the action of the D_2 antagonist raclopride), whereas striatal infusion of the adenosine receptor antagonist theophylline potentiated D_2 mediated effects in extracellular GABA levels (Ferre et al., 1996b).
- At the biochemical level, it was found that the stimulation of striatal A_{2A} receptors changed the binding characteristics of striatal D_2 receptors. Specifically, stimulation of adenosine A_{2A} receptors decreased the affinity of the D_2 receptors for agonists while it didn't change the affinity for antagonists (Ferre et al., 1997).
- In addition to intramembrane interactions, antagonistic regulation between adenosine and dopamine receptors can also exist at the level of second messenger as A_1 and D_2 receptors inhibit adenylyl cyclase and A_{2A} and D_1 activate this enzyme.

- At the behavioural level, and as mentioned above, it has been shown that low doses of A_1 and A_{2A} receptor agonists selectively counteract the motor activating effects induced by D_1 and D_2 receptor agonists, respectively (Ferre et al., 1994).

In fact, in the year 2000 our group reported the first evidence that these adenosine-dopamine interactions could involve heteromeric complexes in the cell membrane. Gines et al. demonstrated the existence of A_1/D_1 complexes in stably cotransfected A_1-D_1 fibroblast Ltk^- cells by means of coimmunoprecipitation. Thus, the A_1/D_1 heteromeric receptor complex may therefore give the molecular basis for the well documented antagonistic A_1/D_1 receptor/receptor interactions found in the neuronal networks of the brain (Fuxe et al., 1998; Franco et al., 2000). However, A_1/D_1 receptor heteromerization in the cotransfected fibroblast cells was strongly reduced by the D_1 receptor agonist treatment, while simultaneous D_1 and A_1 receptor agonist treatment blocked this disruption of the heteromeric complex. Thus, like the study performed between dopamine and somatostatin receptors (Rocheville et al., 2000a), this study shows how agonists alone or simultaneous treatment lead to conformational changes in their respective binding pockets that are transmitted to the heteromeric interface and results in strengthening or disruption of the complex. In addition, the A_1D_1 heteromerization also appeared to have an impact on receptor trafficking. Treatment with the A_1 agonist induced aggregation of both receptors while incubation with the D_1 agonist only induced the clustrering of D_1 receptors and a loss of colocalization.

ADENOSINE AND DOPAMINE RECEPTORS INTERACTION IN PARKINSON DESEASE

The striatum is the main input structure of the basal ganglia and a key component of the motor and limbic systems. On the basis of its afferent and efferent connections the striatum is currently subdivided into two parts, the dorsal and the ventral striatum. Accordingly, the dorsal striatum is mainly represented by the dorsolateral part of the nucleus caudate putamen and the ventral striatum is made of the ventromedial caudate putamen, the nucleus accumbens (with its two compartments, shell and core) and the olfactory tubercle. The basic intrastriatal circuitry is quite simple, only one type of neuron, the projecting GABAergic neuron, constitutes more that 90% of the striatal neuronal population. The GABAergic efferent neurons mainly convey information carried by dopaminergic mesencephalic cells, which are located in the substantia nigra and ventral tegmental area, and by glutamatergic cells located in cortical and limbic areas, like the amygdala and hippocampus (Pollack, 2001). There are two subtypes of striatal GABAergic efferent neurons that give rise to the two dorsal striatal efferent systems, which connect the dorsal striatum with the output structures of the basal ganglia, the substantia nigra pars reticulata, and the entopeduncular nucleus (Alexander and Crutcher, 1990). These are called direct and indirect pathways.

The direct pathway is made of striatonigral and striatoentopeduncular neurons. The indirect pathway consists of the striatopallidal GABAergic neurons, pallido-subthalamic GABAergic neurons, and glutamatergic neurons, which connect the subthalamic nucleus with the output structures. Pallidal GABAergic neurons also project directly to the output structures without using the subthalamic nucleus relay. The striatopallidal GABAergic neurons contain the peptide enkephalin and dopamine receptors predominantly of the D_2 subtype. On the other hand, the striatonigral and striatoentopeduncular GABAergic neurons contain the peptides dynorphin and substance P and dopamine receptors predominantly of the D_1 subtype. Stimulation of the direct pathway results in motor activation and stimulation of the indirect pathway produces motor inhibition. Dopamine, or dopamine agonists, will induce motor activation by activating the direct pathway (acting on stimulatory D_1 receptors) and by depressing the indirect pathway (acting on inhibitory D_2 receptors) (Alexander and Crutcher, 1990).

In Parkinson's disease a preferential degeneration of the nigrostriatal dopaminergic system produces striatal dopamine depletion with the consequent impairment of the functioning of these circuits, which is associated with hypokinesia. Hyperactivity of the GABAergic striatopallidal neurons due to the release from the strong D_2 receptor-mediated tonic inhibitory effects of endogenous dopamine is probably the main pathophysiological mechanism responsible for this hypokinesia (Obeso et al., 2000). The consequent increased neuronal activity of the subthalamic nucleus and the output structures of the basal ganglia (in particular the internal segment of the globus pallidus, which is the counterpart of the rat entopeduncular nucleus in primates) seems to be the functional hallmark of the parkinsonian state, that gives the basis of the surgical treatment in this disease (lesion of the subthalamic nucleus or pallidotomy) (Obeso et al., 2000).

The vast majority of striatal adenosine receptors are located in the medium-sized spiny GABAergic neurons. As has been stated before, one subtype of GABAergic efferent neurons, the striopallidal ones, mainly constitute the indirect output pathway of the striatum while a second subtype, the strionigro-strioentopeduncular neurons, form the direct output pathway. The two subtypes of neurons both contain A_1 adenosine receptors, but only the striopallidal neurons contain A_{2A} receptors in which it is supposed to regulate acetylcholine as well as GABA release. However, of the four adenosine receptor subtypes, the A_{2A} receptor is the focus of intense research within the neurological field, particularly in relation to the hypoactive and hyperactive movement disorders of Parkinson's disease (Richardson et al., 1997) and Huntington's disease, respectively, where the efficacy and chronic use of drug therapies are limited. Indeed, a study by Kanda et al. (Kuwana et al., 1999) reported that oral administration of a selective antagonist of the A_{2A} receptor to hypoactive parkinsonian MPTP-treated marmosets resulted in a modest and sustained increase in motor activity without the onset of abnormal dyskinetic movements. In fact this compound, KW-6002, is now under preclinical observations (Bara-Jimenez et al., 2003).

Therefore, the ability of A_{2A} receptor antagonists to alter motor function becomes relevant for the treatment of Parkinson's disease. Adenosine A_{2A} receptor

antagonists may be able to re-establish the physiological functioning and balance of striatal neurons in parkinsonian patients. Understanding the mechanisms of action of adenosine A_{2A} antagonists in terms of A_{2A}/D_2 interaction would be critical in determining whether A_{2A} antagonists can be used as a therapy in treating Parkinson's disease.

AIMS OF THIS STUDY

Adenosine, acting through its specific receptors, is a key neuromodulator that regulates several neurological functions. Adenosine also affects neuronal differentiation and proliferation but the molecular mechanism of these effects and the receptors involved are not well understood.

1. Since adenosine modulates dopamine actions in the basal ganglia, adenosine receptors are being proposed as potential therapeutical targets for Parkinson's disease. Therefore, the first objective of this thesis has been to characterize the molecular interaction between adenosine A_{2A} receptors and dopamine D_2 receptors and to get insight into the physiological role of adenosine-dopamine receptor-receptor heteromerization.

2. As it is now being accepted, GPCR dimerization is a frequent state for these receptors. Thus, when considering them as therapeutical targets it is important to know their ability to form, not only heterodimers but also homodimers. The second objective for this thesis has therefore been to investigate A_{2A} receptor homodimerization from both a functional and structural perspectives.

3. Finally, the third objective of this thesis has been identify which adenosine receptors mediate the regulation of neuronal differentiation and the identification of the cellular mechanisms involved in this effect.